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NEW ROTIFER BIOASSAYS FOR AQUATIC TOXICOLOGY

FINAL REPORT

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A standard protocol was developed for assessing acute toxicity using the freshwater rotifer *Brachionus calyciflorus*. This protocol has several advantages over existing methods in that test animals are obtained by hatching dormant cysts, the test is highly reproducible, it has a low failure rate, and has good sensitivity to a variety of common pollutants. Cyst age up to 18 months had no effect on test animal response to toxicants. LC50s for copper and pentachlorophenol (PCP) were lower at 10°C and 30°C than at 20°C. *B. calyciflorus* LC50s were a good predictor of *Daphnia magna* and fathead minnow LC50s for eight common toxicants. A standard protocol using *B. calyciflorus* for estimating chronic toxicity of compounds in freshwater has been developed. The rotifer chronic test has several advantages over existing tests including: test animals are obtained from cysts, the test takes only 48 hours to complete, a simple algal food is sufficient for good rotifer reproduction and algae is obtained from a petri dish, the test has good sensitivity to a wide variety of common toxicants. Acute and chronic toxicity of trinitrotoluene has been characterized. The LC50 is 9.1 $mg.L^{-1}$, the chronic value is 3.3 $mg.L^{-1}$, and the acute/chronic ratio is 2.8, suggesting that TNT is only weakly chronically toxic. The effects of temperature on rotifer chronic toxicity over the 20°-30°C range were small.

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FOREWORD

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Final Report

Introduction

Toxicity tests are essential tools for assessing aquatic toxicity because they are sensitive, ecologically relevant indicators. Their application has been limited, however, due to their expense, technical difficulty and long duration. USAMRDC is concerned with the adverse effects on the environment of militarily unique pollutants. New techniques for detection and quantification that are quicker, less costly and more accurate are of great interest. Rotifer cysts represent a new way of obtaining test animals for aquatic tests. Cysts eliminate the necessity of maintaining stock cultures of test animals, a major expense in tests. Cysts can be stockpiled so there is never a problem with test animal availability. A cyst-based rotifer test is technically simpler than existing tests, reducing operator error while increasing speed of results. These improvements are likely to promote the expansion of aquatic bioassays into new applications.

A standardized protocol for conducting a 24 h acute toxicity test using the freshwater rotifer *Brachionus calyciflorus* was developed. This test is unique in that test animals are obtained by hatching dormant cysts, eliminating the need to maintain stock cultures. This off-the-shelf test is conducted in multiwell plates requiring only about 100 ml of test water to execute. In addition to developing a protocol, we have surveyed the acute toxicity of 28 compounds and determined LC50s. This data base has been compared to that available for *Daphnia* and fathead minnows. In general, *B. calyciflorus* has comparable sensitivity to *Daphnia* and fatheads for most classes of toxicants. Pesticides appear to be an exception where rotifers are considerably less sensitive. The effects of test animal age, temperature, cyst age, and salinity were investigated. The results of these studies are reported in two publications (Snell et al. 1989, Snell et al. 1991) which are appended to this report.

Rotifers also are very useful for estimating chronic toxicity. A 48 h test based using reproductive rate as an endpoint was developed and used to investigate the chronic toxicity of several compounds. Chronic toxicity was characterized with only 500 ml of test water. The effects of temperature, food level, and food type

on the chronic toxicity of reference toxicants to *B. calyciflorus* were described (Snell and Moffat1992).

Body

1) Acute Toxicity Test Protocol

Our first objectives were to refine the rotifer acute toxicity test protocol and examine rotifer sensitivity to several toxicants. We have improved the protocol in three ways. First, procedures for rotifer cyst hatching have been adjusted to maximize hatching. Second, techniques for making toxicant stock solutions and dilution series have been simplified to minimize operator error. And third, micropipeting of rotifer neonates has been modified to make transfers easier and less tiring. For a complete description of the acute protocol see the appendix.

2) Sensitivity to Toxicants

Using this refined protocol, we examined the acute toxicity of several compounds with the freshwater rotifer *Brachionus calyciflorus* (BC). LC50 values, coefficients of variation, 95% confidence limits, and sample sizes for 28 toxicants in 24 h acute tests are reported in Table 1. BC sensitivities to these toxicants span about 5 orders of magnitude. With respect to the metals, BC was most sensitive to silver and least sensitive to selenium. Silver, tributyl tin, copper, and mercury were very toxic, whereas zinc, cadmium, nickel, and selenium were only moderately toxic. Aluminum and lead were not toxic at their solubility limits in standard freshwater. BC was sensitive to the pesticide PCP and the herbicide 2,4-D. BC sensitivity to CDNB and SDS was similar to PCP. NaOCl was highly toxic, whereas free ammonia, chloroform, acetone, hexane, dichloroaniline, and diesel fuel were only moderately toxic. BC was not sensitive to phenol, benzene, toluene, or xylene. The pesticides fenitrothion and chlorpyrifos (Dursban) were quite toxic to BC, but the organophosphate trichlorofon was only moderately toxic. The low coefficients of variation for each compound indicate a high degree of reproducibility for BC acute toxicity tests.

Of particular interest to USAMRDC is the compound trinitrobenzene (TNB). We examined the acute toxicity of this compound using the standard rotifer bioassay in four replicate tests. The mean 24 h LC50 was 1.4 $mg.l^{-1}$ with 95% confidence limits of 1.2-1.6 $mg.l^{-1}$. This compares favorably with the sensitivity of *Daphnia magna* and fathead minnows with TNB LC50s of 2.7 and 1.1 $mg.l^{-1}$, respectively (van der Schalie, 1983). Rotifers have comparable sensitivity after only 24 h exposure as compared to the *Daphnia* test where exposure was 48 h and to fathead minnows where exposure was 96 h. The rotifer *B. calyciflorus* therefore is well suited for detecting TNB acute toxicity and can accomplish this faster than existing tests without loss of sensitivity.

Table 1. *Brachionus calyciflorus* LC_{50}s, coefficients of variation (CV), 95% confidence limits (CL) and sample sizes (N) for 28 compounds. All LC_{50}s are in $mg.l^{-1}$ for 24 hr acute toxicity tests except chlorine and chloroform which were 1 hr tests and diesel fuel which is $ul.l^{-1}$.The abbreviations are: NaPCP- sodium pentachlorophenate; CDNB- 1-chloro-2,4-dinitrobenzene; SDS- sodium dodecyl sulfate; 2,4 D - 2,4-dichlorophenoxyacetic acid; and NaOCl- sodium hypochlorite.

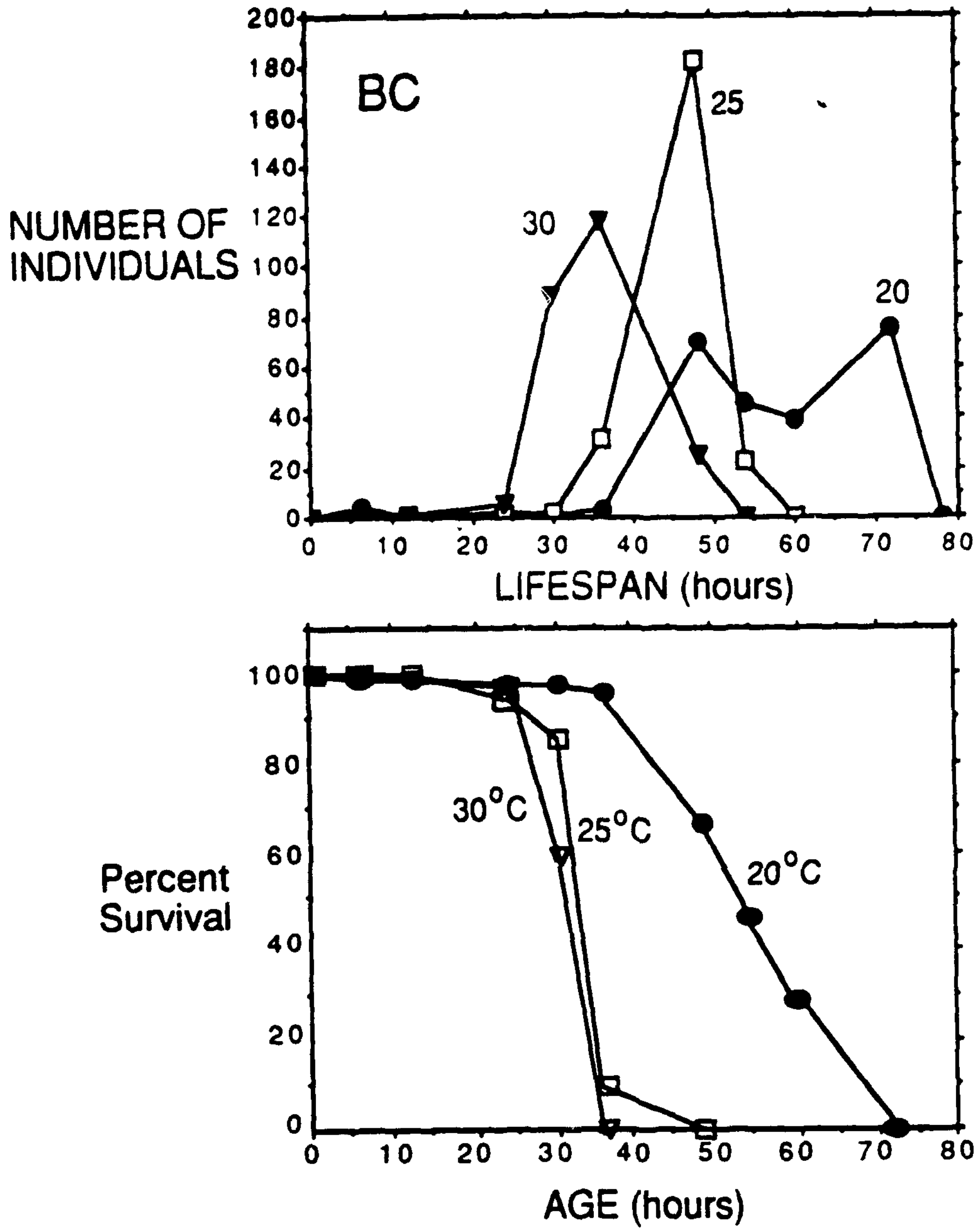
Compound	LC50	CV	95% CL	N
NaPCP	1.2	3.9%	1.1-1.3	5
CDNB	1.3	6.6%	1.1-1.5	4
SDS	1.4	16.6%	1.1-1.6	6
2, 4 D	117	5.1%	102-132	3
acetone	51.1	2.4%	50-52	6
chloroform	2.0	5.9%	1.8-2.2	4
phenol	>150	-	-	1
dichloroaniline	61.5	13.9%	X	X
toluene	113	18.8%	X	X
hexane	68.3	15.2%	X	X
xylene	253	48.8%	X	X
benzene	>1000	-	-	-
Trichlorofon	46.6	13.9%	X	X
Fenitrothion	6.7	10.9%	X	X
Chlorpyrifos	11.9	8.4%	X	X
NaOCl	0.37	3.7%	0.35-0.39	4
diesel fuel	63	-	-	1
free ammonia	4.6	9.6%	4.2-5.1	6
tributyl tin	0.019	12.4%	0.013-0.025	3
copper	0.026	10.8%	0.022-0.030	6
mercury	0.060	4.0%	0.054-0.066	3
cadmium	1.3	11.9%	1.1-1.5	6
zinc	1.3	16.8%	1.1-1.4	6
nickel	4.0	13.7%	3.3-4.6	5
silver	0.0075	3.6%	0.0072-0.0079	5
selenium	16.2	9.5%	13.8-18.7	4
aluminum	>3.0	-	-	1
lead	>4.0	-	-	1

We examined the consistency of test sensitivity by calculating variability in control mortality for the tests in Table 1. Control mortality in 84 acute toxicity tests conducted in our laboratory averaged 2% with a standard deviation of 3%. Mortality ranged from 0-13% with a coefficient of variation of 141%. For the rotifer acute test, control mortality is acceptable when it is less than or equal to 10% of test animals. Only once in 84 tests did control mortality exceed 10%, which yields a failure rate of 1.2%.

Temporal variation in test sensitivity was described as a major problem with acute toxicity tests using the standard test animals *Daphnia pulex* and *Mysidopsis bahia* (Dorn and Rodgers 1989). These authors found significant negative correlations of -0.41 and -0.47 between control mortality and LC50s for *Daphnia* and *Mysidopsis*, respectively. Control mortality ranged from 0-24% for *Daphnia* with a mean of 4% (n=35) and from 0-22% for *Mysidopsis* with a mean of 9% (n=18). Test sensitivity was substantially higher when control mortality was at the higher end of this range. By comparison, BC control mortality ranged from 0-13%, averaged 2%, with a mean failure rate of 1.2%. Using the 10% criteria for excessive control mortality, 14% of the *Daphnia* tests and 39% of the *Mysidopsis* tests failed. We believe that this difference in the amount of control mortality between *Brachionus*, *Daphnia* and *Mysidopsis* acute tests can be attributed to consistency in the physiological condition of animals emerging from cysts. Using neonates hatched from cysts for toxicity tests reduces variability in test sensitivity and reduces failure due to excessive control mortality.

3) Duration of the Rotifer Acute Test

One question we proposed to answer in this project concerned the optimum duration of the acute test. It may be possible to obtain higher sensitivities to toxicants by extending the test to 48, 72 or 96 hours. Experiments were conducted to determine whether control mortality due to starvation limited the duration of the test. A life table approach was taken to characterize survivorship curves for animals in toxicant-free medium at 20°, 25° and 30°C (Figure1).



B

A

Figure 1. Survivorship curves (A) and frequency distributions of *B. calyciflorus* lifespans (B) at 20°, 25° and 30°C in the absence of toxicants.

- There are significant differences in lifespans among the 3 temperatures.
- The shape of the survivorship curve suggests that nearly all mortality is concentrated in the older age classes (negative skew rectangular or Type I curve).
- Mean lifespans in hours ± SEM are: 20°C - 54.8±1.79, 25°C - 46.7±0.13, 30°C - 34.5±0.43

- Test duration is limited by control mortality. Significant control mortality occurs after 36 hr, 30 hr and 24 hr of starvation at 20°C, 25° and 30°C, respectively.

4) Effect of Cyst Age on LC50s

The duration of cyst storage could have an effect on the sensitivity of the resulting hatchlings to toxicants. This possibility was explored in an experiment using the reference toxicant sodium pentachlorophenol (Figure 2).

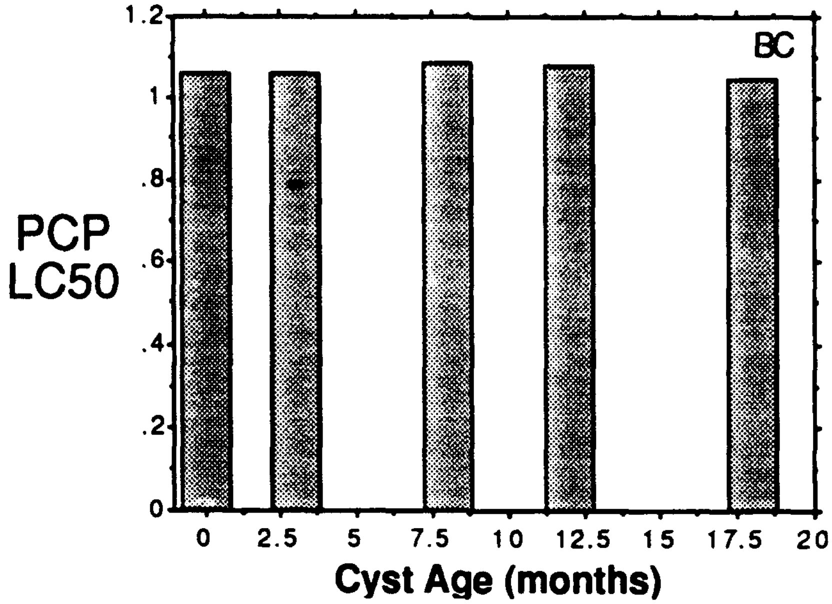


Figure 2. The effect of cyst age on the sensitivity of hatchlings to toxicants. NaPCP LC_{50}s are in mg/l.

- Cyst age ranged from 0 to 18 months
- There were no significant differences in NaPCP LC50s for neonates hatching from cysts of age 0-18 months

5) Effect of Temperature on Rotifer Acute Toxicity

We examined the acute toxicity of copper on the 24 h LC50 of *Brachionus calyciflorus* at temperatures of 10, 15, 20, 25 and 30°C (Figure 3, left). An analysis of variance followed by Sheffe's F test revealed significant effects for copper (ANOVA F=31.7, p<.001). The highest LC50 of 0.031 $mg.l^{-1}$ was recorded at 20°C. The mean LC50 at 10°C (0.018 $mg.l^{-1}$) was significantly lower than 20° as were the LC50s at 25 and 30°C (0.026 and 0.025 $mg.l^{-1}$). This suggests that *B. calyciflorus* has the highest tolerance to copper at 20°C and both higher and lower temperatures increase sensitivity.

A similar analysis was performed on *B. calyciflorus* LC50s for sodium pentachlorophenate (PCP) (Figure 3, right). An analysis of variance followed by Sheffe's F test revealed significant effects for PCP (ANOVA F=4.58, p=.023).

The LC50s for PCP were not significantly different at 15, 20 and 25°C. At 10° and 30° LC50s were significantly lower. Similar conclusions can therefore be drawn for the PCP data as for copper: *B. calyciflorus* has the highest tolerance at intermediate temperatures, whereas both high and low temperatures increase toxicant sensitivity.

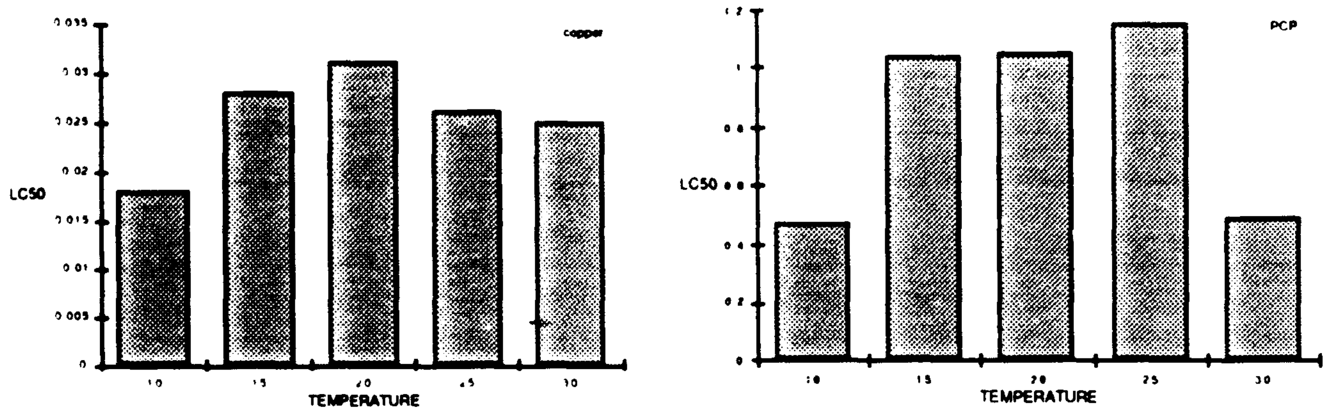


Figure 3. Temperature effects on *B. calyciflorus* LC50s for copper (left) and PCP (right). Each LC50 is the mean of three replicate experiments and are in $mg.l^{-1}$.

6) Comparison of *B. calyciflorus* Sensitivity to *Daphnia* and Fat Head Minnow

In order to compare the sensitivity of *B. calyciflorus* (BC) to that of *Daphnia magna* (DM) and fathead minnows (FHM), we calculated linear regressions for LC50 values for a variety of compounds. Our estimates of 24 hr BC LC50s were regressed on literature values for DM and FHM for the following 12 compounds: SDS, PCP, ammonia, phenol, tributyl tin, copper, cadmium, mercury, lead, zinc, nickel and silver. The BC vs DM regression was significant with $Y= 0.086X + 0.006$ and $R^2=0.999$ (Figure 4, left). For this regression N=8, 1/3 of the compounds (SDS, PCP ammonia and zinc) did not fit the regression model and were omitted.

The BC vs FHM regression also was significant with $Y= 1.48X + 0.859$ and $R^2=0.959$ (Figure 4, right). For this regression also N=8, 1/3 of the compounds (SDS, phenol, nickel and zinc) did not fit the regression model and were omitted. These data suggest that the sensitivity of BC to many compounds is similar to DM and FHM. Approximately 2/3 of the compounds thus far tested have similar toxicities to these species. However, toxicity is very much compound and species specific, so a broader data base is needed before generalizations can be drawn about classes of compounds with similar modes of action.

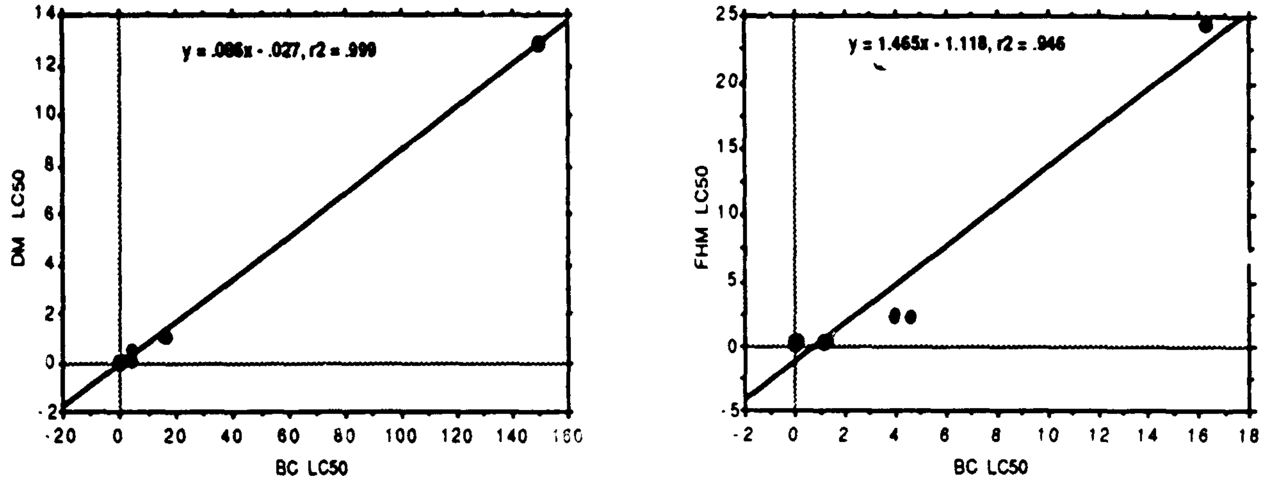


Figure 4. Regressions of *B. calyciflorus* LC50s on those of *Daphnia magna* (left) and fathead minnows (right). All LC50s are in $mg.l^{-1}$.

7) Hatching Response of Brachionus Cysts Exposed to Light for Different Periods

In order to anticipate problems that may be encountered by users of the rotifer test, we investigated the effect of duration of light exposure on cyst hatching. The standard incubation period in light for cyst hatching is 24 h. Exposures as short as 9 h yield cyst hatching equivalent to controls (Figure 5). With only 6 h of light the percenage of cysts hatching in 24 h is cut in half and to 1/8 of controls with 3 h of light. These data illustrate that there is a good margin of safety for duration of illumination required for good cyst hatching.

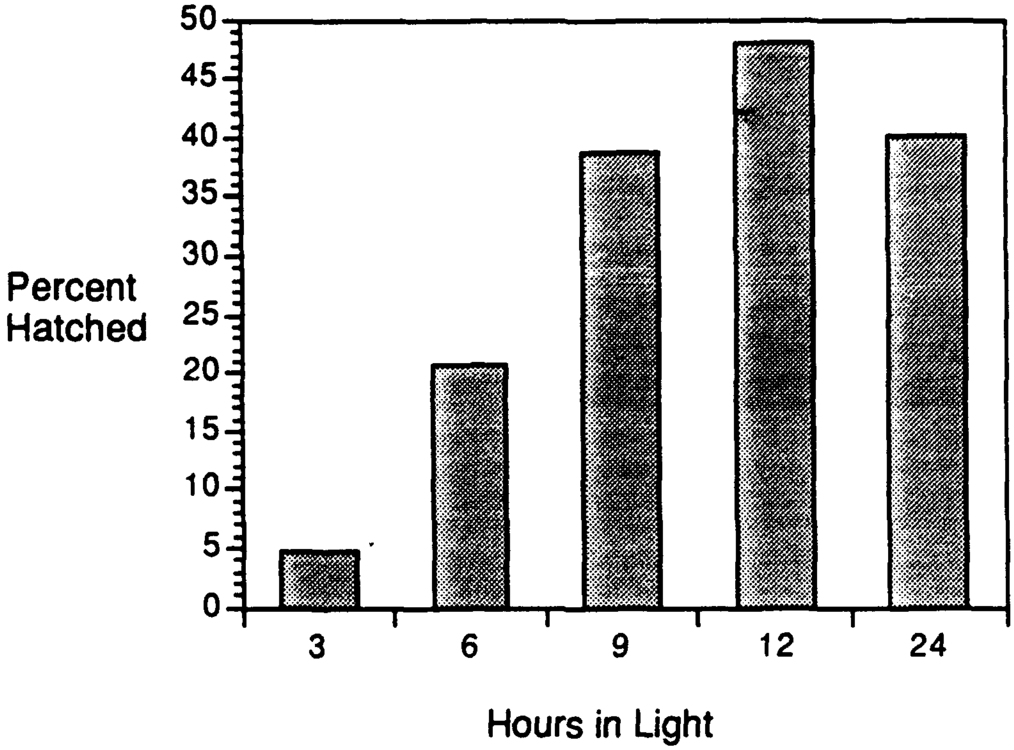


Figure 5. Hatching of *Brachionus* cysts exposed to light for different periods.

8) Development of a 48 h Chronic Toxicity Test using *B. calyciflorus*

We spent several weeks examining a variety of alternatives for assessing chronic toxicity in rotifer populations. From these trials we chose a protocol that provided rapid, consistent results and made sense within the context of the rotifer life cycle. This protocol uses per capita reproductive rate (r) over a 48 h period as an endpoint. The test is initiated by placing 6 neonate rotifers hatched from cysts into a test tube containing 12 ml of standard freshwater and 10^6 *Nannochloris oculata* cells per ml. The tubes are placed in an incubator at 25°C in darkness on a culture rotator revolving at 12 revolutions per hour. After 48 h, the contents of each tube are emptied into a petri dish and the number of rotifer counted. From these data population growth rate r, the intrinsic rate of increase, can be calculated from: $r = \frac{(\ln N_t - \ln N_0)}{T}$ where $N_0 = 6$, N_t = number rotifers after 48 h, and T = 2 days. There are four test concentrations plus a control, each with 7 replicates. This design allows an analysis of variance and Dunnett's to be calculated comparing test concentrations to control and determination of NOEC, LOEC and chronic value. For a complete description of the 48 h reproductive test see the appendix.

A 48 h exposure represents about 30% of rotifer lifespan at 25°C. Although the rotifer test is of short duration, it represents more of test animal lifespan than the 7 day *Cer.ɔdaphnia* chronic test currently widely used in aquatic toxicity assessment. Experiments have shown r to be a sensitive and reproducible

measure of sub-lethal toxicant effects. We also examined egg ratio at 36 h as a possible test criterion. These results were not as reproducible as for r and the fact that r required only 12 more hours of incubation was judged not to be a major disadvantage. In initial experiments we examined both 48 h and 72 h incubations. We were able to obtain the same NOECs, LOECs, chronic values and IC50s in 48 h, so we opted for the shorter incubation.

9) Optimizing the Algal Food for the Rotifer Life Cycle Toxicity Test

We compared three microalgae and one yeast for their ablility to sustain rotifer growth under our experimental conditions. The algae were *Selanastrum capricornutum* (SC), *Kirchneriella cornutum* (KC), *Nannochloris oculata* (NO), all supplied at 100 ug/ml (Figure 6). The yeast (AS) was specially grown for use in aquaculture by Artemia Systems, Inc. and supplied at 10 ug/ml. The highest r was obtained on NO, followed by KC, SC and AS. All algae species were better than the yeast which produced an r value only 35% that of the best algae NO. As a result of these and related experiments, we chose NO as our standard algal food.

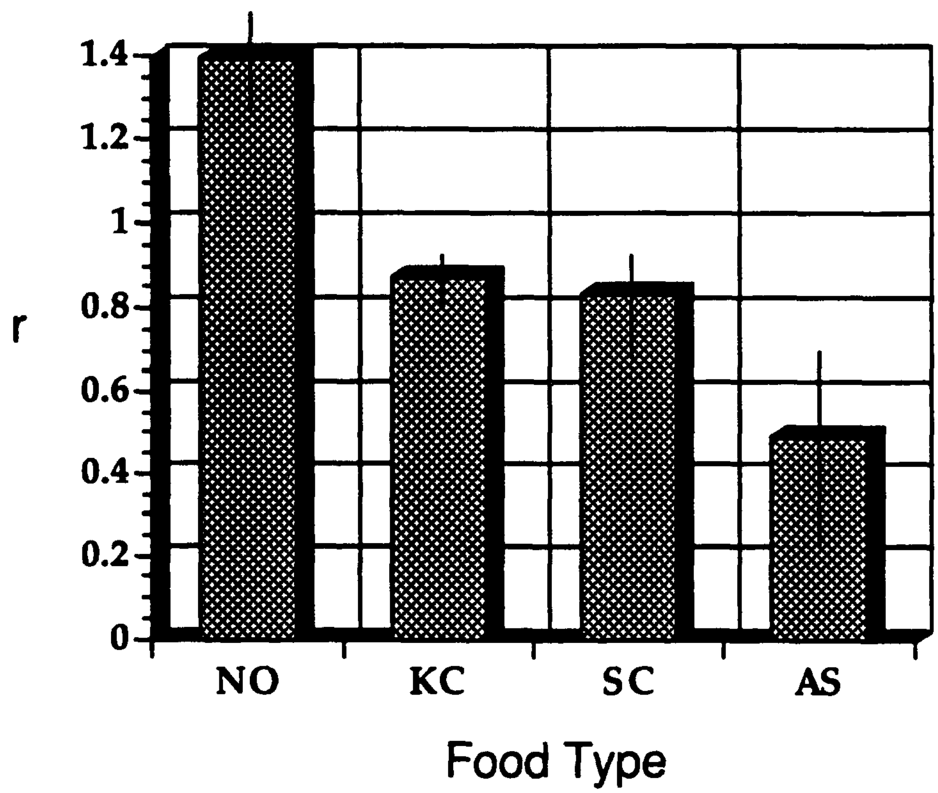


Figure 6. The effects of different algae and yeast diets on rotifer population growth rate. The units for r are offspring per female per day. The food types are *Selanastrum capricornutum* (SC), *Kirchneriella cornutum* (KC), *Nannochloris oculata* (NO), all at 100 ug/ml and yeast (AS) at 10ug/ml. Vertical lines on top of columns are standard errors for seven replicate measures.

To optimize the level of NO for the rotifer life cycle toxicity test, we examined several NO concentrations ranging from 50,000 to 3 million cells per ml. The strategy here was to choose the lowest food level yielding good rotifer population growth. A food level of 3 million NO cells per yielded an r of 1.02 ± 0.06, whereas a food level of 13 million NO cells per ml yielded an r of 1.4 ± 0.13. By choosing the lower food level we obtained good r, yet we minimized any algae-toxicant interactions that may occur during the test.

We investigated several concentrations of yeast in hopes that some level would yield a high enough r in toxicant-free controls to use in the life cycle test (Figure 7). The highest r obtained was 0.49 ± 0.16, which was only 48% of that obtained on a diet of 3 million algal cells per ml. In addition to low r, the yeast diet produced higher variance among experiments than algae. Consequently, we concluded that yeast was not an acceptable food for the rotifer life cycle test.

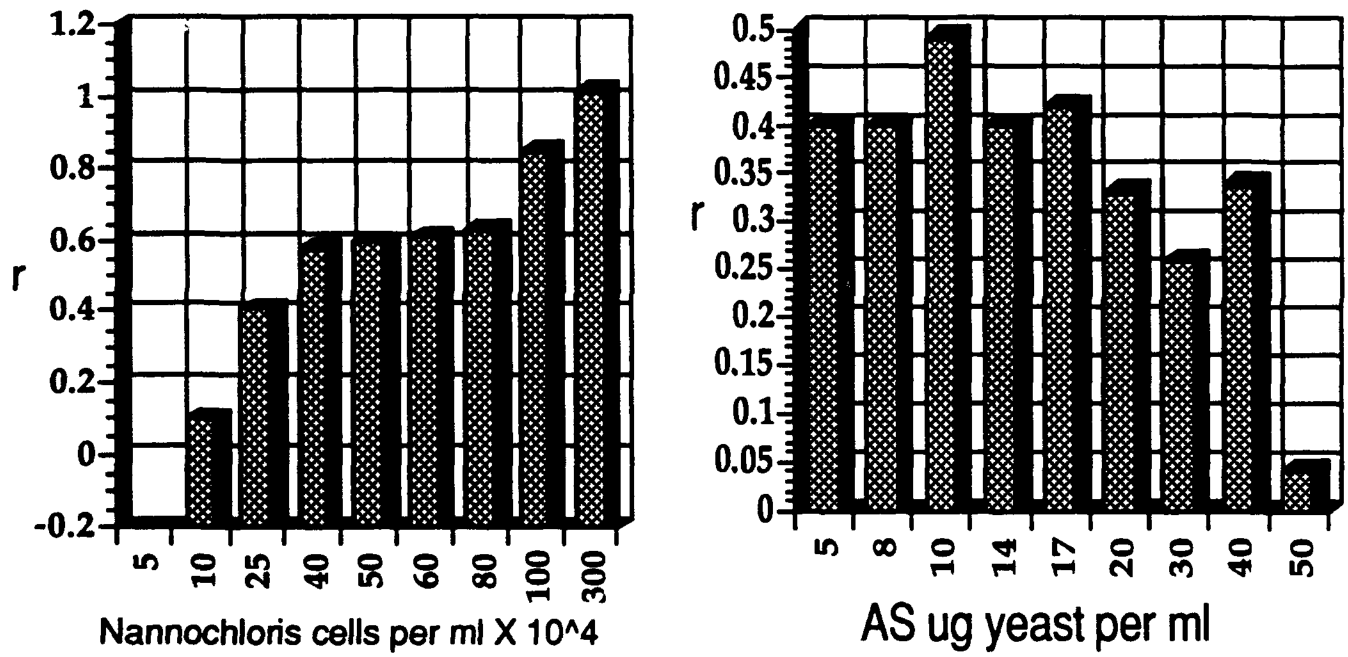


Figure 7. Effects of algal (left) and yeast (right) food levels on rotifer population growth. The units for r are offspring per female per day.

10. Inert Diets for the Rotifer Reproductive Test

Several inert feeds have been examined for their ability to support rotifer reproduction and population growth. If an inert feed were suitable for use in toxicity testing, algae culture would be eliminated as a requirement for the rotifer test. This represents a great savings of time and costs for routine toxicity screening. Algae cells can be maintained alive on agar petri dishes for several months and reconstituted in suspensions when needed. Plate *Nannochloris oculata* cells yield population growth rates equivalent to those on fresh live algae (Figure 8). Spray dried *Nannochloropsis salina* cells are a

newly available product that is also convenient to use. These dried cells have a confirmed shelf life of six months and probably will last considerably longer. Suspensions of spray dried *Nannochloropsis salina* yielded rotifer growth rates that were not significantly different from fresh live cells.

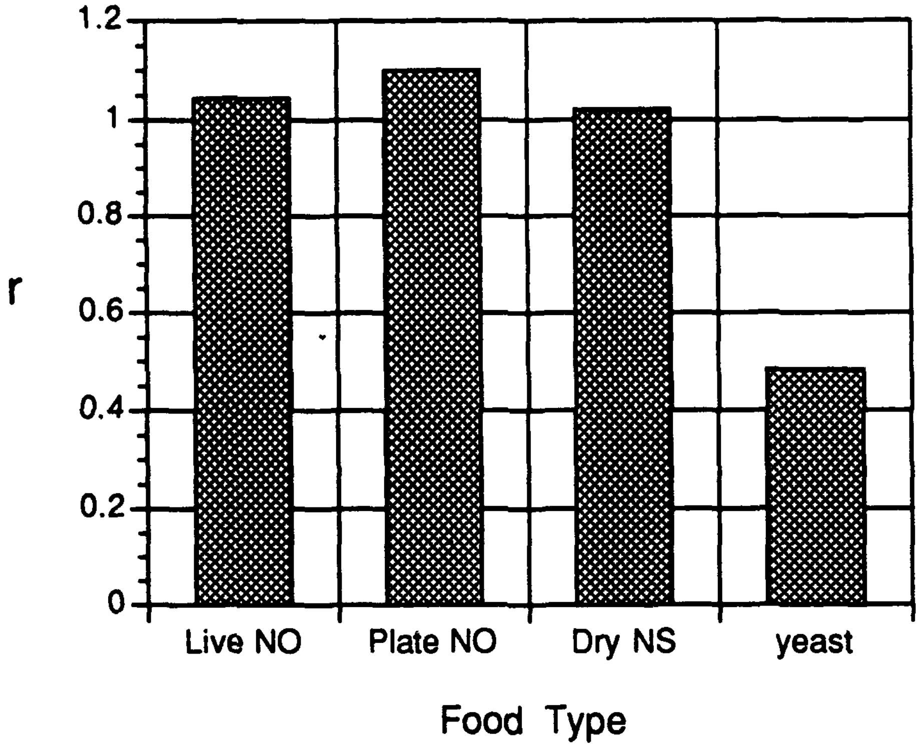


Figure 8. *Brachionus* population growth rate (r) on different diets. r is offspring per female per day. Live NO is live *Nannochloris oculata* cells, plate NO is *Nannochloris oculata* cells maintained on an agar plate for 2.5 months, dry NS is six month old spray dried *Nannochloropsis salina* cells, and yeast is a special yeast product for rotifer culture from Artemia Systems, Ltd. (Belgium).

The ability of most animals to withstand the stress of toxicant exposure is closely linked to the quality of their diet. It is important to demonstrate that the response of rotifers to toxicants after feeding on inert diets is similar to that on a live algae diet. The chronic values and IC50s for PCP of rotifers fed fresh live algae and plate algae are not significantly different by ANOVA (Figure 9). The chronic value of rotifers fed dry *Nannochloropsis salina* cells was 0.24 mg/L, higher than the 0.14 value for rotifers fed fresh live or plate algae. The IC50 for rotifer fed dry *Nannochloropsis salina* cells was higher at 0.54 mg/L than the 0.24 mg/L of rotifers fed fresh live or plate algae. Although these differences were significant by ANOVA, they are relatively small in magnitude. It therefore appears that rotifers fed dry algae diets respond similarly to PCP as those fed live algae.

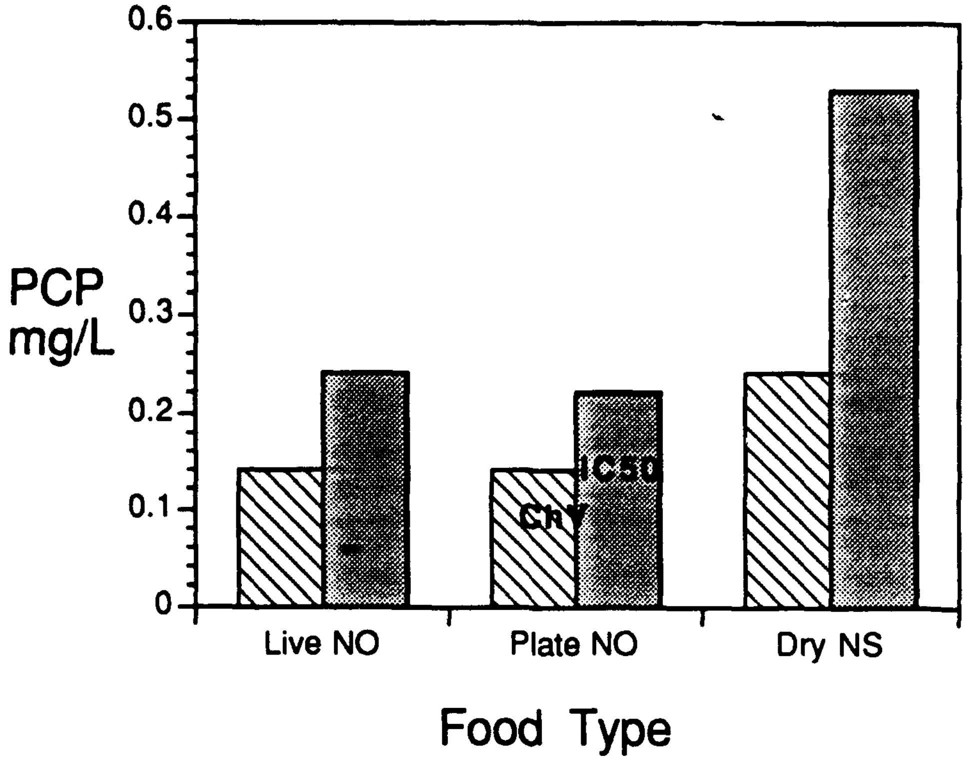


Figure 9. A comparison of sensitivity of rotifers fed fresh live *Nannochloris oculata* cells (live NO), *Nannochloris oculata* cells maintained on an agar plate (plate NO), and spray dried *Nannochloropsis salina* cells (dry NS).

11) Chronic Toxicity of Single Chemicals Characterized Using the Rotifer Test

A standardized 48 h rotifer reproductive test was developed and used to examine the chronic toxicity of several single chemicals to *Brachnious calyciflorus* (BC) (Table 2). NOECs and LOECs were determined by analysis of variance, chronic value is the geometric mean of the NOEC and the LOEC, IC50s and LC50s were determined by linear regression. 2,4 dimethyl phenol, TNT, and Diazinon all had similar NOEC values. NOECs for phenol and xylene were similar, but substantially higher than those of the first group. BC was quite sensitive to the organophosphate pesticide Chlorpyrifos with an NOEC of 0.23 mg/L. Acute/chronic ratios for the eight compounds ranged from 2 for TNT to 33 for Chlorpyrifos.

Table 2. The chronic toxicity of eight single chemicals to *B. calyciflorus*. All table values are nominal concentrations mg/L. NOEC- no observed effect concentration, LOEC- lowest observed effect concentration, ChV - chronic value, IC50 - 50% inhibition concentration, LC50 - 50% lethal concentration.

Compound	NOEC	LOEC	Chronic value	IC50	LC50	acute/ chronic
PCP	0.11	0.19	0.14	0.27	1.2	18
phenol	25	43	32	59	780	13
2,4 dimethyl phenol	2	4	2.8	8.6	208	24
trinitrotoluene	2.3	5	3.3	4	9.1	2
xylene	20	40	28	99	253	3
cadmium	0.04	0.08	0.06	0.07	1.3	18
Diazinon	8	13	10	11	31	3
Chlorpyrifos	0.23	0.33	0.27	0.36	12	33

The sensitivity of the 48 h chronic reproductive test using *B. calyciflorus* was compared to the sensitivity reported for Daphnia and Ceriodaphnia. Table 3 compares the NOECs for pentachlorophenol and cadmium in µg/L. Brachionus is about three times more sensitive to PCP than *Daphnia* and *Ceriodaphnia*, but these latter two are 16-80 times more sensitive to cadmium than *Brachionus*.

Table 3. A comparison of NOECs for *Brachionus*, *Daphnia* and *Ceriodaphnia*. Table values are NOECs in µg/L. *Brachionus* endpoint was r in a two day test; *Daphnia* and *Ceriodaphnia* endpoints were total young per female in 7 day tests (from Winner, R. W., 1988)

Compound	Brachionus	Daphnia	Ceriodaphnia
PCP	110	300	300
cadmium	40	2.5	0.5

12) The Acute and Chronic Toxicity of Trinitrotoluene

The acute and chronic toxicity of a trinitrotoluene (TNT) sample supplied and quantified by the USAMRDC was determined. On February 21, 1990 we received approximately 100 ml of a sample determined to be 100 mg TNT/L by HPLC in USAMRDC laboratories (Tom Shedd, personal communication). We conducted acute and chronic toxicity tests with *B. calyciflorus* using this sample and returned it within three weeks to USAMRDC for reanalysis.

Reanalysis revealed that the sample was 92.8 ppm. The concentration of the stock solution that we used in our dilution series for acute and chronic tests was therefore quite stable. A sample from the highest TNT concentration in one of our tests was also checked using HPLC by USAMRDC. Our nominal TNT concentration at the beginning of the test was 7 mg/L and the measured value after the test was 5.6 mg/L.

The mean LC50 for TNT to *B. calyciflorus* was 9.1 mg/L and the mean chronic value in the life cycle test was 3.3 mg/L (Table 4). The acute/chronic ratio shows that mortality occurs at TNT concentrations 2.8 times higher than those significantly depressing reproduction. Dose-response data for both acute and chronic toxicity are presented in Figure 10. For mortality, the reponse is sigmoid, with a linear portion between 5 and 11 mg/L. For reproduction, a slight enhancement in reproduction is observed at 1 mg/L TNT followed by a linear decline to 7 mg/L.

Table 4. Three replicate life cycle toxicity tests with TNT.

statistics	replicate 1	replicate 2	replicate 3	mean	coeff. var.
NOEC	1.0	3.0	3.0	2.3	-
LOEC	5.0	5.0	5.0	5.0	-
ChV	2.24	3.87	3.87	3.33	28.3%
IC50	3.19	4.64	4.21	4.01	18.6%

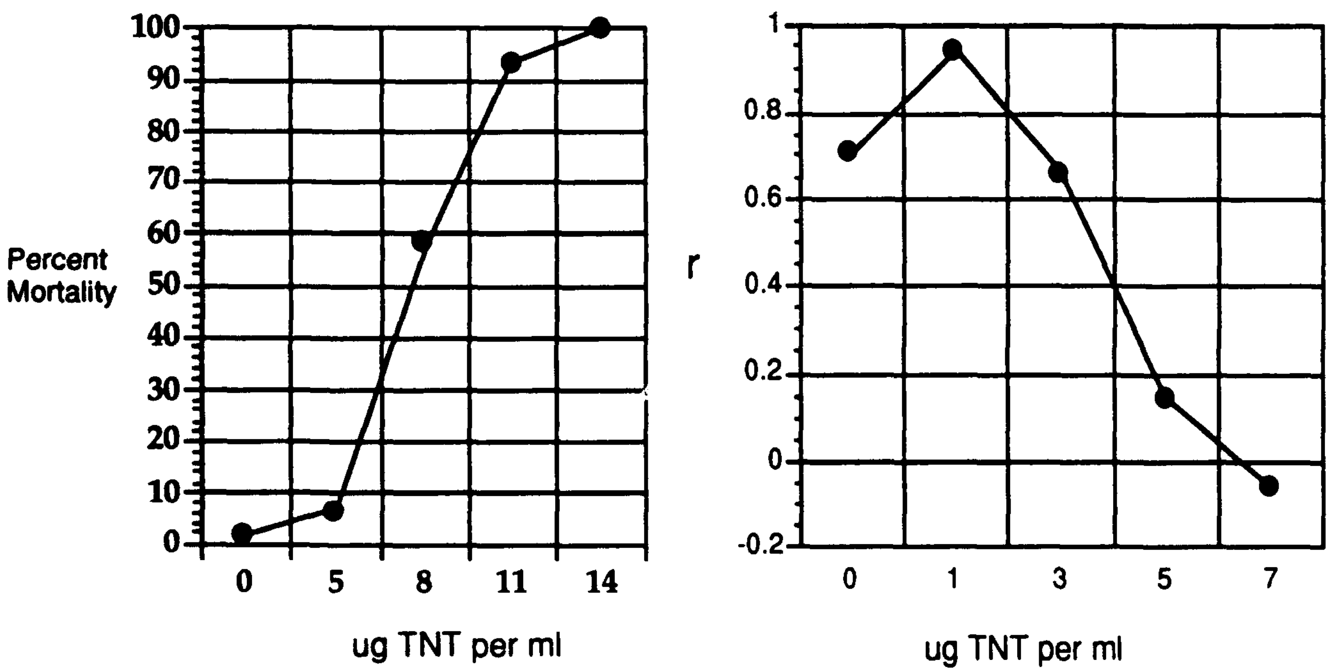


Figure 10. Dose-response curves for acute (left) and chronic (right) toxicity of TNT. Each data point is the mean of three replicate estimates.

13) Schedule of Reproduction during 48 h Chronic Tests

It is important to describe the schedule of reproduction by individual females in the test tubes of a 48 h chronic test. The endpoint of the test is r and is determined by counting the total number of animals after 48 h. The schedule of births to maternal females is not observed in the test tubes during a test, but this schedule is helpful in understanding the population dynamics behind a toxic response. Consequently, we executed an experiment to follow the reproductive output of individual females by observing them at one hour intervals over a 48 h incubation period. The experimental conditions were standard for the rotifer chronic test: 25°C, moderately hard EPA dilution water, 3 million *Nannochloris* cells/ml, and darkness. Tubes were inoculated with 2 ml of algal suspension and a single female, which was examined hourly for 48 h and the offspring removed. These observations allowed us to determine when eggs were extruded and when they hatched. The complete schedule is illustrated in Figure 11.

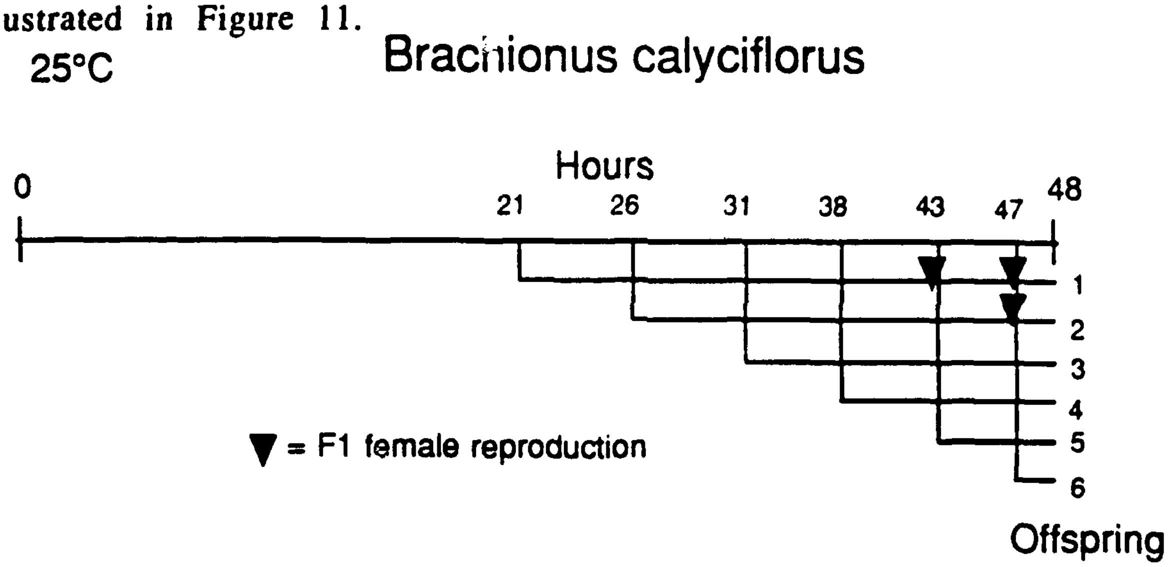


Figure 11. Schedule of reproduction during a 48 h rotifer chronic test. Maternal females give birth to their first offspring at 21 h, their second at 26 h, etc. Inverted triangles indicate reproduction by F1 females.

Maternal females gave birth to their first offspring at age 21 h. Subsequent births occurred at approximately 5 hour intervals until the test was terminated at 48 h. The first born F1 female gave birth to an offspring at 42 h and another at 47 h. The second F1 female was born at 26 h and gave birth to an offspring at 47 h. The total reproductive output during the 48 h test was therefore 6 offspring from the maternal female and 3 from the F1 females for a total of 9 offspring. These data should aid us in interpreting the results of toxicant suppression of reproductive activity in the rotifer chronic test.

14) Temperature Effects on Chronic Toxicity

The effects of temperature on chronic endpoints is important to characterize. An examination of the response of the rotifer chronic test to temperature should also provide some clues as to how lab results might relate to field results at different temperatures. Standard conditions for the rotifer chronic test were: 25°C, moderately hard EPA dilution water, 3 million *Nannochloris* cells/ml, and darkness. We also conducted the test at 20° and 30°C to examine temperature effects. Tubes were inoculated with 12 ml of algal suspension and six *B. calyciflorus* females. After 48 h, population counts were made and r calculated as $\ln N_t - \ln N_0 / 2$ days. Differences among the three temperature treatments were examined by one-way analysis of variance (Table 5).

Table 5. Analysis of variance using temperature (20°, 25°, 30°C) as the independent variable and IC50 for PCP or hexavalent chromium as the dependent variable. This tests the null hypothesis that temperature has no effect on IC50.

PCP ANOVA

Source	df	Sum Squares	Mean Squares	F	P
between grps	2	.05	.025	1.48	0.30
within grps	6	.10	.017		
total	8	.15			

hex chromium ANOVA

Source	df	Sum Squares	Mean Squares	F	P
between grps	2	19.1	9.5	2.9	0.13
within grps	6	19.6	3.3		
total	8	38.7			

No significant differences were observed in IC50 values for either PCP or hexavalent chromium over the temperature range 20° to 30°C (Figure 11). These results suggest that over the range tested, *B. calyciflorus* appears to have broad temperature tolerance and its reponse to toxicants is not different at 20°C and 30°C. The mean IC50s for this experiment are plotted in Figure 12. Although the IC50 for both PCP and hex chromium appear to be slightly higher at 30°C, these means are not significantly different according to the analysis of variance.

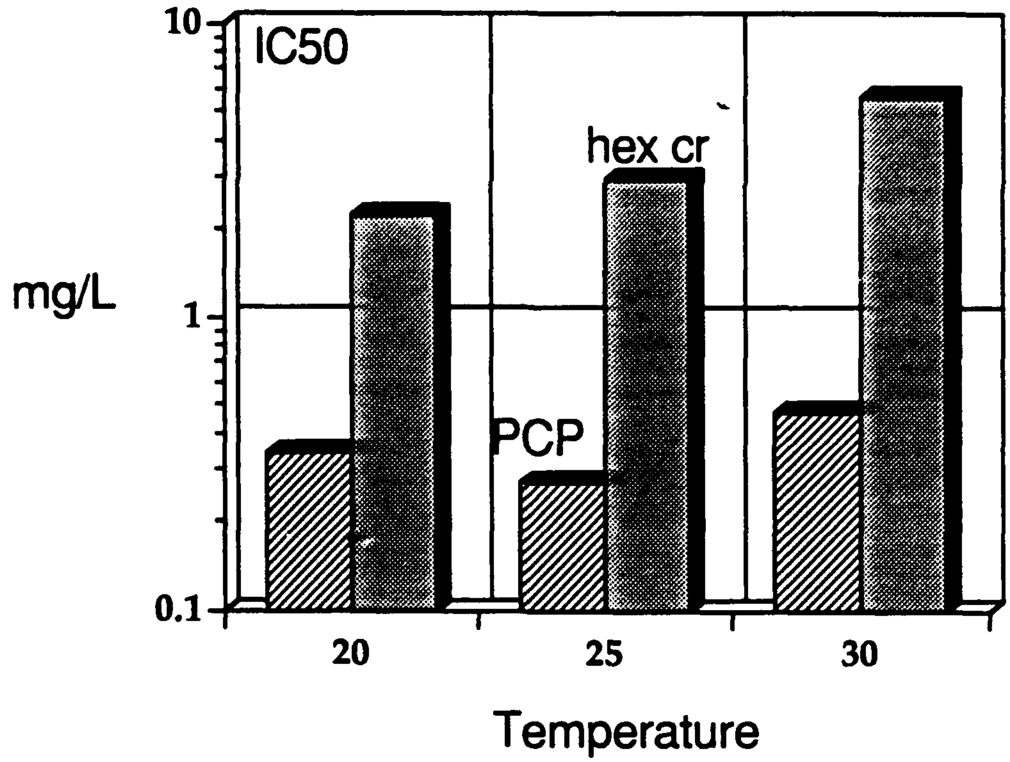


Figure 12. The effects of temperature on the chronic toxicity of pentachlorophenol (PCP) and hexavalent chromium (hex cr) to *B. calyciflorus*. IC50 (y axis) is in mg/L and temperatures (x axis) are 20°, 25° and 30 °C.

15) Bioavailability of Reference Toxicants in the Chronic Rotifer Test

When algae is present, toxicants might bind to the algal cells, thus reducing their bioavailability to the test animals. To be useful for chronic tests, reference toxicants must be shown to have the same bioavailability in the presence and absence of algae. We examined the concentration of two reference toxicants in the presence of algal cells using analytical techniques to determine their concentration. For pentachlorophenol (PCP) we used a spectrophotometric method described by Haskins (1951) and for hexavalent chronium we used the diphenylcarbohydrazide method described in ASTM (1989). The nominal and actual concentrations for both toxicants are listed in Table 6.

Table 6. Comparison of nominal and actual concentrations of two reference toxicants. Toxicant concentrations are in mg/L. The test was a standard 48 h rotifer chronic test after which the algae were removed by filtration and the filtrate analyzed for toxicants.

Toxicant	Nominal conc mg/L	actual conc before test	% difference	actual conc after test	% difference from nominal
PCP	0.40	0.36	11.1	0.44	10.0
Cr^{+6}	2.00	2.04	2.0	2.14	7.0

It therefore appears that both PCP and hexavalent chromium are suitable reference toxicants for rotifer chronic tests because their bioavailability is not affected by the presence of algae.

Conclusions

1) A standard protocol was developed for assessing acute toxicity using the freshwater rotifer *Brachionus calyciflorus.*
2) This protocol has several advantages over existing methods in that test animals are obtained by hatching dormant cysts, the test is highly reproducible, it has a low failure rate, and has good sensitivity to a variety of common pollutants.
3) Cyst age up to 18 months had no effect on test animal response to toxicants. Lc50s for copper and pentachlorophenol (PCP) were lower at 10°C and 30°C than at 20°C.
4) *B. calyciflorus* LC50s were a good predictor of *Daphnia magna* and fathead minnow LC50s for eight common toxicants.
5) A standard protocol using *B. calyciflorus* for estimating chronic toxicity of compounds in freshwater has been developed.
6) The rotifer chronic test has several advantages over existing tests including: test animals are obtained from cysts, the test takes only 48 hours to complete, a simple algal food is sufficient and is obtained from a petri dish, the test has good sensitivity to a wide variety of common toxicants.
7) Acute and chronic toxicity of trinitrotoluene has been characterized. The LC50 is 9.1 $mg.L^{-1}$, the chronic value is 3.3 $mg.L^{-1}$, and the acute/chronic ratio is 2.8, suggesting that TNT is very weakly chronically toxic.
8) The effects of temperature over 20-30°C range were small. No significant differences in IC50s for PCP and hexavalent chromium were detected at 20°, 25° and 30°C test temperatures.

9) The presence of algae in chronic tests might affect bioavailability of toxicants. PCP and hexavalent chromium concentrations were quantified before and after tests. No change in toxicant concentration suggested that the toxicity of these compounds was not mitigated by algal cells. Consequently, PCP and hexavalent chromium are good candidates for reference toxicants.

Publications

Snell, T. W. and G. Persoone. 1989. Acute toxicity bioassays using rotifers. I. A test for brackish and marine environments with *Brachionus plicatilis*. Aquatic Toxicology. 14: 65-80.

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Snell, T. W., B. D. Moffat, C. Janssen and G. Persoone. 1991. Acute toxicity tests using rotifers: IV. Effects of cyst age, temperature, and salinity on the sensitivity of *Brachionus calyciflorus*. Ecotox. & Environ. Safety 21:308-317.

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Brachionus calyciflorus Freshwater Bioassay Protocol

Objective: The rotifer test is a standardized, simple and cost-effective bioassay for screening toxicity in freshwater. Using the rotifer *Brachionus calyciflorus*, an acute toxicity test is executed in 24 hours with a sensitivity comparable to that of other aquatic invertebrates.

1. Standard Freshwater Medium Preparation :
   Carefully add 96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4$, and 4 mg KCl to one liter of research grade deionized water. Mix well on a magnetic stirrer and adjust pH to 7.5 with KOH or HCl. This is a moderately hard standard freshwater medium with hardness of 80-100 mg $CaCO_3$ per liter and alkalinity of 60-70 mg per liter.

2. Rotifer Cyst Hatching:
   Rotifer cyst hatching should be initiated 24 hours before the start of a toxicity test. Place about 40 ml of standard freshwater into a petri dish, empty the contents of one vial of rotifer cysts into the water and rinse the vial to remove all cysts. Incubate the petri dish at 25°C in light of 1000-4000 lux for 16-18 hours. Make sure that most cysts are submerged during the incubation by rinsing the sides of the hatching dish with a pipet. Hatching should start after 15 hours and 1-2 hours later the dish should be taken out of the incubator for transfering rotifers to test plates. Cooler temperatures, low or high pH, elevated hardness and alkalinity can delay hatching. If hatching is delayed, check cysts hourly to insure collecting test animals within 2 hours of hatching. It is important to obtain 0-2 hour old animals to start the bioassay because there is no feeding during the test. The effects of starvation begin to cause mortality after about 32 hours at 25°C. If rotifers are older than 32 hours at the end of the test, excessive control mortality may result, invalidating the bioassay.

3. Preparing a dilution series:
   As the rotifers are hatching, prepare a dilution series of the test compound or effluent according to standard methods (see USEPA, 1985).

4. Filling the control wells:
   The bioassay is conducted in 24-well polystyrene plates (Corning 25820). Notice that these plates are labeled as columns 1-6 across and rows A-D down. Pipet 1 ml of standard freshwater into the 4 wells in column 1 (the left most column) of the test plate. This treatment is freshwater without toxicant and will serve as the control. Working from the lowest concentration, pipet 1 ml of the test compound solution into each of the 4 wells of column 2 of the test plate. Repeat this procedure for the wells in columns 3-6.

5. Adding the rotifers:
Beginning with the control, use a micropipet to transfer about 50 rotifers from the hatching dish into the fourth well of column 1 (column 1, row D) of the test plate. Now transfer 10 rotifers from this fourth well into each of the wells A-C in column 1. Take care to minimize the transfer of medium along with the rotifers. This protocol washes the neonates in the appropriate test solutions before they enter the test wells, thus reducing dilution of the test solutions during rotifer transfer. Repeat this two-step transfer procedure for columns 2-6. This design yields a control and five test concentrations, each with three replicates of ten animals.

6. Incubation and scoring of the test plate:
Incubate the completed test plate at 25°C in darkness. After 24 hours, score each well for the number of live and dead rotifers. Rotifers are considered dead if they do not exhibit any external or internal movement in 5 seconds of observation. Complete the following table:

Test Concentration	# alive	#dead	% mortality
0 (control)			
1			
2			
3			
4			
5			

For this test to be valid, control mortality must be 10% or less. An LC_{50} can be calculated from these data using any of the standard methods (see USEPA, 1985). Every 5-10 assays, a reference test should be run to confirm proper adherence to protocol and test sensitivity. When using sodium pentachlorophenate as a reference toxicant, the LC_{50} value should be within the 95% confidence limits of 1.1 - 1.3 $mg.l^{-1}$.

References

Snell, T. W. and G. Persoone. 1989. Acute toxicity tests using rotifers. II. A freshwater test with *Brachionus rubens*. Aquatic Toxicology. 14: 81-92.

US Environmental Protection Agency 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. W.H. Peltier and C.I. Weber (eds.), EPA-600/4-85-013. 216 pgs.

Brachionus calyciflorus Two Day Chronic Reproductive Test

Objective: The rotifer two day reproductive test is a simple, standardized test for assessing chronic toxicity in freshwater. This test yields data similar to the seven day *Ceriodaphnia* test and has comparable sensitivity.

Overview: Rotifer cysts are hatched by incubating in standard freshwater for 16 hours at 25°C in light. Newly hatched rotifers are used to initiate a chronic toxicity test which is based on population growth rate (r) over 48 hours. The data produced permits calculation of an NOEC, LOEC, chronic value and IC50.

Schedule of Work: The rotifer chronic test fits conveniently into a nine to five workday and 5 day work week. One day prior to setting up a test, rotifer cysts are placed into a hatching dish at about 5 pm and incubated for 15 hours. By 8 am the next morning hatching begins and technicians have about 2 hours to prepare the algal suspension, toxicant dilution series and transfer rotifers to the test tubes. The test tubes are placed on a rotator and scored after 48 hours for population growth rate. From start to finish, approximately 66 hours elapse from incubation of cysts to calculation of the results.

Rotifer Cyst Hatching

1) The test is initiated by hatching rotifer cysts which are supplied dry in a small vial. The hatching medium is a standard freshwater prepared according to the following recipe:

   Carefully add 96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4$, and 4 mg KCl to one liter of research grade deionized or distilled water. Mix well on a magnetic stirrer and adjust pH to 7.5 with 1 N KOH or HCl. This is a moderately hard standard freshwater medium with hardness of 80-100 mg $CaCO_3$ per liter and alkalinity of 60-70 mg per liter.

2) Rotifer cyst hatching should be initiated 15-16 hours before the start of a toxicity test. To initiate hatching, place about 40 ml of standard freshwater into a petri dish. Glass petri dishes or tissue culture grade polystyrene dishes are preferable, because some common types of disposable polystyrene dishes might contain compounds toxic to rotifers.

3) Empty the contents of one vial of rotifer cysts into the water and rinse the vial to remove all remaining cysts. Incubate the petri dish at 25°C in light of 1000-4000 lux for 15-16 hours. Make sure that most cysts are in contact with water during the incubation by rinsing the sides of the hatching dish with a pipet.

4) Hatching should start after about 15 hours and within 2 hours the dish should be taken out of the incubator for transfering rotifers to the test tubes. Cooler temperatures, low or

high pH, elevated hardness and alkalinity can delay hatching. If hatching is delayed, check cysts hourly to insure collecting test animals within 2 hours of hatching.

5) If cysts are not used for testing immediately, they may be stored for several months in a refrigerator. Cysts should be used within one year of acquisition.

Preparing the Algal Suspension

1) To obtain *Nannochloris* cells for one chronic test, cut the algae disk in half with a spatula, remove half and place it into a 100 ml beaker or petri dish. Algal cells are rinsed from the agar base and resuspended in standard freshwater in four steps:
   a) Pour approximately 50 mls of standard freshwater over the agar, washing the algae cells off.
   b) Gently rub the remaining algae cells from the agar using a clean finger.
   c) Carefully remove the agar base, taking care not to break it apart.
   d) Mix the algal suspension gently on a magnetic stirrer (approximately 120 rpm) using a small stir bar(1/2" x 5/16"). Use algae promptly, do not stir for more than 30 min.

2) Quantify algae cell density using a Neubauer slide (hemacytometer) according to the following protocol:
   a) Place one drop of algal suspension on a Neubauer slide and examine at 400x magnification.
   b) There should be 10 to 20 cells per smallest square on the slide. If dilution is required, record the dilution factor and dilute a small sample (1 ml) of the algae stock.
   c) Count enough small squares so that the total number of algae cells counted is at least 300.
   d) Calculate the mean number of cells per small square and multiply by 400. This gives the total number of cells in the entire grid.
   e) Multiply the result in part d by 10,000 to obtain the number of cells per ml in the original stock. If dilution was required, multiply part d by 10,000 and the dilution factor.

3) Calculate the volume of algal stock needed to make 100 ml of a 3.0×10^6 cells/ml algal suspension.

4) Calculate toxicant dilutions for 100 ml treatment volumes.

5) Add approximately 75 ml of standard freshwater to a 100 ml volumetric flask followed by the required volume of algal stock.

6) Add the required volume of toxicant to the volumetric flask (omit this step for the control treatment).

7) Immediately bring to 100 ml volume with standard freshwater.

8) Pour the suspension into a 150 ml beaker and stir gently on a magnetic stirrer. Use algae promptly, do not stir for more than 30 min.

9) Repeat steps 5 through 8 for each treatment. Typically, a rotifer chronic test has 1 control and 4 toxicant or effluent concentrations.

10) Pipette 12 mls of the control algal suspension into each of 7 replicate test tubes. Disposable 16 X 150 mm glass test tubes work well.

Adding the Rotifers

1) Rotifers are small animals approximately 250 µm in length which is about 1/4 the size of newborn *Daphnia*. Do not panic when you first see rotifers under the microscope at 10X magnification. Their small size and slow swimming speed has some advantages for capturing and manipulation. Newly hatched rotifers are white, so they are most visible on a dark background. If your microscope has a darkfield setting, this is the best type of illumination. A dark background also can be obtained with most microscopes by adjusting the angle of illumination and its intensity.

2) Since they are moderately phototactic, rotifers tend to congregate around the edges of the hatching dish. There are several ways to make rotifer manipulation easier. The micropipet should be grasped like a pencil, using the index finger and thumb to provide pressure on the bulb. This position provides the best control and produces the least fatigue, but any position may be used if it feels comfortable. The bulb should be squeezed gently to provide adequate suction. It will take practice to develop a feel for just the right pressure. After about 15 minutes practice, most people become sufficiently skilled at collecting, counting and transfering rotifers rapidly to successfully complete the test.

3) To initiate the test, transfer 6 newly hatched rotifers (neonates) into each control test tube (from step 10 above), cap and immediately place the tubes on a rotator in a 25°C incubator in darkness. Rotation should be about 12 revolutions per hour. Record the time at which the neonates are placed into the control treatment as the beginning of the 48 hour incubation period.

4) Pipette 12 mls of the lowest toxicant suspension from the stirrer into the next set of seven replicate test tubes. Transfer 6 neonates into each tube, cap and immediately place the tubes on the rotator. Repeat this step until the remaining toxicant suspensions have been inoculated with rotifers.

Scoring and Calculation of Results

1) Remove test tubes from the rotator after 48 hours, empty the contents into a petri dish, and count the number of animals per tube.

2) Calculate r, the intrinsic rate of increase, for each tube according to:

$$r = \frac{\ln N_t - \ln N_o}{T}$$

Where: N_t = number of rotifers in tube after 2 days
N_o = initial number of rotifers in tube (6)
T = 2 days
ln = natural logarithm

3) An analysis of variance and Dunnett's test can be calculated to compare each toxicant concentration to the control. This is a one-way ANOVA with 5 treatments, each with 7 replicates. From these data NOEC, LOEC and chronic values can be calculated.

4) An IC50 can be calculated by linear regression of toxicant concentration on r. The regression equation, if significant, can be used to calculate the toxicant concentration yielding 50% reduction in r (IC50) as compared to controls.

Sample Data Sheet

Conc	tube	Nt	r	Conc	tube	Nt	r	Conc	tube	Nt	r
contr	1			c1	1			c2	1		
contr	2			c1	2			c2	2		
contr	3			c1	3			c2	3		
contr	4			c1	4			c2	4		
contr	5			c1	5			c2	5		
contr	6			c1	6			c2	6		
contr	7			c1	7			c2	7		
mean				mean				mean			
CV				CV				CV			

Conc	tube	Nt	r	Conc	tube	Nt	r	Conc	tube	Nt	r
c3	1			c4	1			c5	1		
c3	2			c4	2			c5	2		
c3	3			c4	3			c5	3		
c3	4			c4	4			c5	4		
c3	5			c4	5			c5	5		
c3	6			c4	6			c5	6		
c3	7			c4	7			c5	7		
mean				mean				mean			
CV				CV				CV			